AD_____

Award Number: DAMD17-97-1-7001

TITLE: The Symptomatic Persian Gulf Veterans Protocol: An Analysis of Risk Factors with an Immunologic and Neuropsychiatric Assessment

PRINCIPAL INVESTIGATOR: Warren D. Blackburn, M.D.

CONTRACTING ORGANIZATION: Vistar, Incorporated
Birmingham, Alabama 35233

REPORT DATE: January 2000

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

# REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188



| 1. AGENCY USE ONLY (Leave blank) | 2. REPORT DATE | 3. REPORT TYPE AND DATES COVERED |
|---|---|---|
| | January 2000 | Annual (23 Dec 98 - 22 Dec 99) |

**4. TITLE AND SUBTITLE**
The Symptomatic Persian Gulf Veterans Protocol: An Analysis of Risk Factors with an Immunologic and Neuropsychiatric Assessment

**5. FUNDING NUMBERS**
DAMD17-97-1-7001

**6. AUTHOR(S)**
Warren D. Blackburn, M.D.

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**
Vistar, Incorporated
Birmingham, Alabama 35233

**E-MAIL:**
warren.blackburn@med.va.gov

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**10. SPONSORING / MONITORING AGENCY REPORT NUMBER**

**11. SUPPLEMENTARY NOTES**

**12a. DISTRIBUTION / AVAILABILITY STATEMENT**
Approved for public release; distribution unlimited

**12b. DISTRIBUTION CODE**

**13. ABSTRACT** ***(Maximum 200 Words)***

Veterans returning from the Persian Gulf reported a myriad of complaints. Several hypothesis have been generated to account for these complaints and include exposure and infection with mycoplasma or related organisms and alterations in immunological responsiveness. To investigate these possiblities, we are studying Gulf War Veterans who have complaints referable to at least 2 organ systems that have been otherwise unexplained. Control groups include in theatre veterans who are asymptomatic and non-theatre veterans who are applying for disablity. We have identified 177 symptomatic veterans, 193 asymptomatic controls, and 312 disability controls. To date, we have collected samples from 50 of the symptomatic controls, 28 asymptomatic controls, and 12 of the disability controls. Evaluation of peripheral blood mononuclear cells, urine and throat cultures samples for the presence of mycoplasma or ureaplasma organisms by culture and PCR revealed no discernable significant differences. Similarly, no significant differences have been detected in *in-vitro* responsiveness.

**14. SUBJECT TERMS**
Gulf War Syndrome

**15. NUMBER OF PAGES 22**

**16. PRICE CODE**

| 17. SECURITY CLASSIFICATION OF REPORT | 18. SECURITY CLASSIFICATION OF THIS PAGE | 19. SECURITY CLASSIFICATION OF ABSTRACT | 20. LIMITATION OF ABSTRACT |
|---|---|---|---|
| Unclassified | Unclassified | Unclassified | Unlimited |

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

## FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

___ Where copyrighted material is quoted, permission has been obtained to use such material.

___ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

___ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

N/A In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

N/A For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

N/A In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

N/A In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

12/20/95

PI - Signature Date

TABLE OF CONTENTS:

**5. Introduction:**

The overall project objective is to determine if there are fundamental immunologic abnormalities in Gulf War veterans which might be associated in a case control study with their service in the Gulf. In order to determine this, the following specific goals will be addressed: 1) evaluate Persian Gulf Veterans with symptoms to determine if there are *in vitro* abnormalities of immunologic tests; 2) evaluate Persian Gulf War veterans to determine if there is evidence to suggest increased exposure to mycoplasma.

**6. Body:**

This study will determine whether specific *in-vitro* immunological abnormalities or evidence of differing exposure to mycoplasma can be detected in GW participants who are symptomatic, but in whom no specific diagnosis has as yet been made. As controls, we are assessing GW veterans who are asymptomatic and non-theater veterans who have applied for disability.

To date: 1,541 Persian Gulf War veterans have been evaluated in this study who were also enrolled in the Birmingham registry. Charts have been available for 1,347 and they have been carefully reviewed and have had second physician review. One hundred and seventy-seven have illnesses involving more than two organ systems that have not been accounted for with a defined illness. Samples have been obtained from 50 of these.

Second, we have received and have categorized the list of veterans who served in the Persian Gulf from the Birmingham catchment area. This is the group of individuals who will represent our in-theater, asymptomatic individuals and will be drawn from a list of 1,383 in theater Alabama veterans. There are 193 in the Birmingham area, of whom we have evaluated 28 samples.

Third, we identified individuals in the Birmingham catchment area who were not in theater, but who have applied for disability. These individuals represent the second control group we have identified as a listing of 312 veterans and we have obtained samples from 12.

Peripheral blood mononuclear cells, throat swabs and urines have been evaluated for the presence of mycoplasma or ureaplasm. Each sample was evaluated by a) direct culture; b) direct PCR; and c) by PCR with a universal mycoplasma primer. Mycoplasma or ureaplasma were detected frequently in urine and throat swabs, however no significant differences have been noted between the three groups

Data are expressed as number positive/evaluable samples.

| SAMPLE | Asymptomatic | Symptomatic | Disability |
|---|---|---|---|
| **PB mononuclear cells** | | | |
| Culture | 0/25 | 0/42 | 0/9 |
| Direct PCR | 0/27 | 1/49 | 1/9 |
| PCR with primer | 0/24 | 0/45 | 0/8 |
| **Throat** | | | |
| Culture | 14/26 | 14/46 | 6/12 |
| Direct PCR | 0/24 | 0/47 | 0/12 |
| PCR with primer | 22/26 | 32/47 | 2/12 |

| **Urine** | | | |
|---|---|---|---|
| Culture | 7/26 | 13/47 | 6/12 |
| Direct PCR | 0/26 | 0/49 | 0/12 |
| PCR with primer | 4/26 | 5/47 | 2/12 |

Testing has been done and is in progress on a cohort of Gulf War veterans and controls for the following *in vitro* immunological functions:

1) NK cell activity as a measure of primitive capacity to respond to foreign stimuli;
2) lymphocyte proliferative capacity using T-cell polyclonal activators (concanavalin A [Con A] and phytohemagglutinin [PHA]);
3) primary immune responses using allogeneic stimulator cells in mixed leukocyte reactions (MLRs); and
4) secondary immune response using the recall antigens tetanus toxoid, *Candida albicans* extract, and the Anthrax Vaccine.
5) B-cell polyclonal activator pokeweed mitogen-induced immunoglobulin (Ig) production; and
6) cytokine expression in order to detect functional abnormalities in antigen presenting cells, type-1 T-helper cells, and type-2 T-helper cells.

Blood has been collected and processed on 90 subjects and controls in the immunology laboratory. Immune function tests numbered 1 through 4 above were attempted on all subjects; however, *n* values less than 90 indicate that the volume of blood was not sufficient for all tests to be performed on a given patient(s), the white blood cell (WBC) yield was not sufficient on a given patient(s), or a lab error resulted in no reportable data for a given patient(s). Samples (blood and/or stimulated cell supernatants) for immune function tests numbered 5 and 6 above are being stored at -70°C pending batch analysis.

Information required to interpret attached spreadsheets is as follows:

**NK cell function**: activity is reported in lytic units derived from dose-response analysis

**Mitogen tests**: Con A 1, 2, and 3 are Concanavalin A at 10, 5, and 2.5 μg/ml, respectively, and PHA 1, 2, and 3 are Phytohemagglutinin at 10, 5, and 2.5 μg/ml, respectively.

**Mixed leukocyte reactions:** Auto MLR 1 ,2, and 3 are 4 x $10^6$, 2 x $10^6$, and 1 x $10^6$ subject or control PBMCs, respectively, cultured without further additions, whereas Allo MLR1 ,2, and 3 are 4 x $10^6$, 2 x $10^6$, and 1 x $10^6$ subject or control PBMCs, respectively, co-cultured with 2 x $10^6$ mitomycin C-treated pooled allogeneic stimulator cells.

**Antigen tests**: TT 1, 2, and 3 are Tetanus Toxoid antigen at 5, 2.5, and 1.25 μg/ml, respectively; AV 1, 2, and 3 are Anthrax Vaccine antigen (in the form of alum-free Protective Antigen) at 2.0, 1.0, and 0.5% of supplied stock, respectively; and C.Alb 1, 2, and 3 are *Candida albicans* antigen at 1.25, 0.625, and .031 KPU/ml, respectively.

| | Symptomatic | | | Asymptomatic | | | Disability | | |
|---|---|---|---|---|---|---|---|---|---|
| Test | number | mean | Std Error | number | mean | Std Error | number | mean | Std Error |
| NK cell | 29 | 60.1 | 14.5 | 20 | 79 | 17.5 | 10 | 44.5 | 8.2 |
| Con A 1 | 42 | 126.9 | 17.3 | 26 | 89 | 15.9 | 8 | 150.5 | 30.1 |
| Con A 2 | 42 | 89.8 | 12.9 | 26 | 63.9 | 12.3 | 8 | 107.1 | 22.6 |
| Con A 3 | 42 | 57.9 | 9.5 | 26 | 41.2 | 9.5 | 8 | 67.4 | 17.9 |
| PHA 1 | 42 | 206.2 | 21.0 | 26 | 176.1 | 20.3 | 8 | 205.8 | 37.1 |
| PHA 2 | 42 | 177.9 | 15.4 | 26 | 149.8 | 17.8 | 8 | 189.9 | 31.1 |
| PHA 3 | 42 | 123.3 | 12.1 | 26 | 98.5 | 14.8 | 8 | 134.1 | 21.8 |
| AutoMLR1 | 40 | 4.3 | 1.2 | 25 | 2.7 | 0.6 | 8 | 2.6 | 0.7 |
| AutoMLR2 | 40 | 2.1 | 0.4 | 25 | 1.5 | 0.2 | 8 | 1.5 | 0.2 |
| AutoMLR 3 | 40 | 1.5 | 0.2 | 25 | 1.4 | 0.1 | 8 | 1.4 | 0.3 |
| AlloMLR1 | 40 | 60.7 | 14.6 | 25 | 66.6 | 20 | 8 | 64.5 | 20.5 |
| AlloMLR2 | 40 | 34.8 | 9.1 | 25 | 26.4 | 9.8 | 8 | 58.2 | 25.2 |
| AlloMLR3 | 40 | 10.9 | 3.1 | 25 | 11.4 | 4.4 | 8 | 33.6 | 19.5 |
| TT 1 | 39 | 35.5 | 9.8 | 26 | 27.8 | 8.1 | 8 | 33.5 | 14.5 |
| TT 2 | 39 | 31.8 | 8.3 | 26 | 21 | 7.9 | 8 | 29.1 | 12.4 |
| TT 3 | 39 | 31.5 | 8.3 | 26 | 24.2 | 8.2 | 8 | 32.1 | 15 |
| AV 1 | 18 | 1.4 | 0.2 | 9 | 1.1 | 0.2 | 2 | 1.1 | 0.2 |
| AV 2 | 18 | 2.1 | 0.7 | 9 | 1.8 | 0.6 | 2 | 2 | 0.3 |
| AV 3 | 18 | 2.1 | 0.8 | 9 | 1.4 | 0.4 | 2 | 2 | 0.4 |
| C.Alb 1 | 31 | 13.3 | 4.1 | 25 | 3.9 | 1.1 | 8 | 15.2 | 7.3 |
| C.Alb 2 | 31 | 14.0 | 3.8 | 25 | 6.3 | 2.2 | 8 | 19.8 | 8.9 |
| C.Alb 3 | 31 | 13.0 | 3.8 | 25 | 4.5 | 1.5 | 8 | 13.6 | 5.7 |

When immunological parameters measured in blood samples taken from symptomatic subjects are compared with those from asymptomatic controls or disability controls, no statistical differences are found using unequal variance T tests and correcting for multiple comparisons.

An interesting note is that the rationale for inclusion of a disability control group in these comparisons was supported by the finding that the response to *Candida albicans* antigen was originally significantly different ($p=0.036$ for C.Alb 1, and $p=0.044$ for C.Alb 2) between the symptomatic and asymptomatic Gulf War veterans groups. However, when these comparisons are adjusted for multiple comparisons, the data are no longer significantly different. Furthermore, when the symptomatic Gulf War veterans results were compared with the disability control group, the data were not significantly different ($p=0.833$ for C.Alb 1, and $p=0.942$ for C.Alb 2) with or with adjusting for multiple comparisons.

**7. Key Research Accomplishments:**

-Our study has not documented any significant differences in symptomatic GW veterans when compared to controls in evidence of exposure to mycoplasma

-Our study has not documented any significant differences in *in-vitro* immunological testing in symptomatic GW veterans when compared to controls.

**8. Reportable Outcomes:**


1. Campo L. Larocque P. La Malfa T. Blackburn WD. Watson HL. Genotypic and phenotypic analysis of Mycoplasma fermentans strains isolated from different host tissues. Journal of Clinical Microbiology. 36(5):1371-7, 1998.
2. Everson MP, Kotler S, and Blackburn WD. Stress and Immune Dysfunction in Gulf War Veterans Annals NY Academy of Science 876: 413-418, 1999.
3. *Is There Immune Dysregulation in Symptomatic Gulf War Veterans? Michael P. Everson, Ke Shi, Peggy Aldridge, Alfred A. Bartolucci, and Warren D. Blackburn, Jr. VA Medical Center and UAB, Birmingham, Alabama, USA, 1999.


*Presented as an abstract in Germany, summer 1999:

**9. Conclusions:** Study is still ongoing and we are continuing to collect samples.

**10. References:** N/A.

JOURNAL OF CLINICAL MICROBIOLOGY, May 1998, p. 1371–1377
0095-1137/98/$04.00+0

Vol. 36, No. 5

# Genotypic and Phenotypic Analysis of *Mycoplasma fermentans* Strains Isolated from Different Host Tissues

LAURA CAMPO,[1] PATRICK LAROCQUE,[1] TIZIANA LA MALFA,[1] WARREN D. BLACKBURN,[2] AND HAROLD L. WATSON[1]*

*Department of Microbiology, University of Alabama at Birmingham,[1] and Birmingham Veterans Administration Medical Center,[2] Birmingham, Alabama*

Received 17 December 1997/Returned for modification 22 January 1998/Accepted 18 February 1998

**A correlation was found between the expression of a specific *Mycoplasma fermentans* surface antigen (Pra, proteinase-resistant antigen) and the site of isolation of the organism from the infected host. Strains which expressed Pra were most frequently associated with cells of bone marrow origin, and strains which lacked expression of Pra were most commonly isolated from the respiratory tract, genital tract, and arthritic joints, i.e., epithelial cell surfaces. Pra was previously shown to be resistant to degradation by proteinases and was hypothesized to play a protective role at the organism surface and perhaps to influence which host tissue site was colonized by the organism. The methods used for this phenotyping scheme required isolation and growth of the mycoplasma in quantities sufficient for immunoblot analysis using monoclonal antibodies. We wanted to determine a more rapid and less cumbersome technique to supplement this method for determining the Pra phenotype directly in clinical specimens. Here we describe PCR studies to investigate the movement of a previously identified *M. fermentans* insertion sequence (IS)-like element. These data showed a correlation between a specific IS genotype and the Pra$^+$ phenotype. Production of a 160-bp product using a single set of IS-based primers was associated with expression of Pra. The genomic IS location resulting in the 160-bp product was determined by using Southern blot analysis and was found to be a stable insertion site characteristic of genotype I strains. Additional analyses of sequences within and flanking the IS insertion sites revealed another pair of PCR primer sites which resulted in the consistent production of a 450-bp amplicon. The stability of this site was dependent on the absence of the IS-like element between the primer sites. The production of this 450-bp amplicon correlated with the Pra mutant phenotype and was characteristic of genotype II strains. The data showed that the sequence within the IS may be unstable and that reliable genotyping sequences are more easily found in the stable genomic sites which flank the IS element.**

First mistakenly identified as a novel AIDS-associated virus (18), *Mycoplasma fermentans* incognitus, during the ensuing years, was considered to be a possible cofactor contributing to acceleration of the progression of this immune disorder (8, 16, 20–22, 29). Immediately following the first reports, several laboratories began probing into this question, but to date, the hypothesis of a mycoplasma-AIDS association remains unproved. However, these studies have added much to our basic knowledge of mycoplasmas. It has been documented that *M. fermentans*, as well as some other mycoplasmas, can occur intracellularly, which was only an occasionally reported and unproved observation prior to these studies. The ability of specific subpopulations of these organisms to survive within host cells could account, at least in part, for the characteristic chronicity of mycoplasmal disease, as well as for the frequent difficulty of isolation by culture. Additionally, an impressive volume of literature is accumulating which describes the induction of various cytokines by mycoplasma infection (3, 5, 15, 27, 28, 30, 37). The potential to alternately stimulate or suppress the immune system would impart a distinct advantage to any pathogen (or commensal organism) attempting to survive in the hostile and changing environment of an infected host.

Subsequent to the initial isolation of strain incognitus, *M. fermentans* was identified as the likely etiologic agent of an acute fatal disease in otherwise healthy adults (17). No other infectious agents were found. A similar wasting syndrome leading to death was reported in silvered leaf monkeys after experimental infection with this same agent (19). Many years prior to these recent studies, *M. fermentans* was isolated from bone marrow of leukemic patients (24) and other reports associated it with rheumatoid arthritis (2, 36). These reports prompted further investigations, including some experimental studies with animal models (9, 10, 26). None of these studies resulted in data proving a cause-and-effect relationship between *M. fermentans* infection and human disease. In fact, early serologic studies provided evidence that antibodies to this organism are common in adolescents and young adults (32).

Therefore, *M. fermentans* has been tentatively associated with disease throughout its history but the precise etiologic role of *M. fermentans* in disease remains unclear. This is, in part, due to the frequently unsuccessful attempts to isolate mycoplasmas in general by routine culture methods (6) and to the presence of individuals harboring the organism without signs of disease. Even though many cases have resulted in isolation of *M. fermentans* and each isolate has been assigned a new strain designation, there has been no attempt to assign molecular or functional characteristics to these strains which might assist in determining if there is a characteristic or group of characteristics which associate with specific diseases, or at least with sites of isolation.

In the present study, we were interested in defining methods to determine if specific strains exhibit characteristics which are more frequently associated with particular tissue sites within an infected host. We tested whether monoclonal antibodies (MAbs) developed against *M. fermentans* antigens could dis-

* Corresponding author. Present address: Infectious Diseases Research, Lilly Research Laboratories, Lilly Corporate Center, Indianapolis, IN 46285-0438. Phone: (317) 277-0131. Fax: (317) 276-1743. E-mail: Watson_Harold_L@Lilly.com.

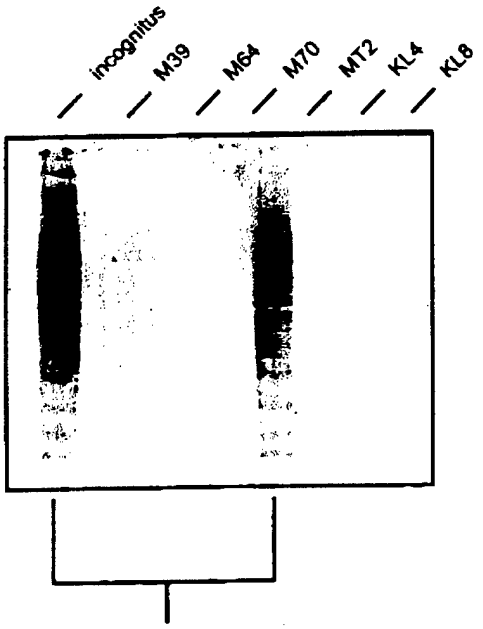


FIG. 1. Pra phenotypes of representative *M. fermentans* strains. Organism proteins were separated by SDS-polyacrylamide gel electrophoresis and then transferred to nitrocellulose. Reactivity with MAb 1A2.6 was visualized by using peroxidase conjugates. Strains incognitus and MT2 show the characteristic $Pra^+$ pattern, while the $Pra^-$ strains show no reaction with the MAb. Results for all strains are summarized in Table 1.

TABLE 1. Relationships among the genotypes, phenotypes, and sources of isolation of different *M. fermentans* strains

| Strain[a] | PCR product size (bp)[b] | Genotype determined by Southern blotting[c] | Immunoblotting result[d] | Specimen origin |
|---|---|---|---|---|
| M51 | Diffuse | II | − | Respiratory tract |
| M39 | 80 | II | − | Respiratory tract |
| M52 | Diffuse | II | − | Respiratory tract |
| M64 | 226 | II | − | Respiratory tract |
| M73 | Diffuse | II | − | Respiratory tract |
| M70 | <80 | II | − | Respiratory tract |
| KL8 | >860 | II | − | Rheumatoid arthritis, joint |
| KL4 | >860 | II | − | Rheumatoid arthritis, joint |
| PG18 | Diffuse | II | − | Human genital tract |
| 12406 | >1,100 | II | − | Human genital tract |
| E10 | 160 | I | + | Leukemic patient, blood |
| 16700 | 160 | I | + | Human genital tract |
| AOU | 160 | I | + | AIDS patient, blood |
| Z62 | 160 | I | + | Leukemic patient, blood |
| Incognitus | 160 | I | + | Kaposi's sarcoma |
| AMSO | 160 | I | + | Lymphocyte culture |
| MT2 | 160 | I | + | Human lymphocyte |
| Elliman | 160 | I | + | Hybridoma |
| DEPB | 160 | I | + | AIDS patient, blood |
| 48429 | 160 | I | + | Cell culture |
| K7 | 160 | I | + | Leukemic patient, blood |

[a] See Materials and Methods for descriptions.
[b] PCR was performed with the RS primers shown in Fig. 4.
[c] Southern blotting was performed with ORF-1 and ORF-2 probes.
[d] Immunoblotting was performed with MAb 1A2.6. −, negative; +, positive.

tinguish between isolates of *M. fermentans* to determine a possible correlation between the expression of these factors and the site of isolation. We also conducted the same correlative assessment for the chromosomal distribution of the *M. fermentans* insertion sequence (IS)-like element, hypothesizing a role for this potentially mobile element in the repression or activation of a specific gene expression.

## MATERIALS AND METHODS

**Sources of isolation.** The *M. fermentans* strains evaluated in this study were isolated from various sources (see Table 1). Strains were obtained as follows: E10 (24) and K7 (25) were obtained from W. H. Murphy; 16700, 12406, and DEPB were from the University of Alabama at Birmingham; AOU was from Luc Montagnier (Pasteur Institute, Paris, France); Z62 was from P. Hannan (Beecham Labs) (24); incognitus was from Shyh Lo (National Institute of Allergy and Infectious Diseases [NIAID]) (17, 18, 21); AMSO was from Ann Robinson (Laboratory of Immune Genetics, NIAID); MT2 was from W. J. Leonard and N. F. Halden (National Institute of Child Health and Human Development) (11); Elliman was from H. Elliman (University of Illinois, Chicago); 48429 was from Andy Lewis (NIAID); M51, M39, M52, M64, M73, and M70 were from R. Dular (Public Health Laboratory, Ottawa, Ontario, Canada); KL4 and KL8 were from P. Hannan (Beecham Labs); and PG18 was from Klieneberger-Nobel, Lister Institute, London, United Kingdom.

**Organisms and growth conditions.** Cultures of *M. fermentans* were grown in SP-4 medium (mycoplasma broth base, tryptone [Difco], peptone [Difco], arginine, phenol red [1%], DNA, and antibiotics for SP-4, supplemented with 10% fetal bovine serum, CMRL 1066, yeast extract, yeastolate, and glucose). The cultures were incubated at 37°C. Samples were harvested and washed in phosphate-buffered saline at pH 7.3. DNA was purified in accordance with standard protocols (phenol-chloroform-isoamyl alcohol) and concentrated by ethanol precipitation. DNA preparations were RNase A treated (Sigma).

**Southern blotting and DNA hybridization.** For Southern blotting of the *M. fermentans* strains, 0.2 μg of genomic DNA was digested with 5 U of *Hin*dIII (Promega, Madison, Wis.) for 2 h at 37°C. The samples were electrophoresed on 0.8% Tris-borate-EDTA agarose gels (50 V for 16 h) and transferred to 1× Hybond $N^+$ nylon membranes. DNA was UV cross-linked to the membrane in a UV Stratalinker 1800 (Stratagene). After prehybridization, the membranes were hybridized with 5′-end $\gamma$-$^{32}$P-labeled oligonucleotide probe RW006 (5′-GCT GTG GCC ATT CTC TTC TAC GTT-3′; see Fig. 3a) and probe ORF-1 (5′-GGA AAA CTC TTA TTC AGC C-3′; see Fig. 3b), located within the insertion sequence transposase gene and open reading frame 1 (ORF-1). Hybridization was performed at 42°C for 1 h in Rapid hyb (Amersham) hybridization buffer and followed by one washing in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS) for 20 min at room temperature and two washings in 0.5× SSC–0.1% SDS for 15 min each at 45°C. DNA hybrids were visualized by autoradiography using Kodak XAR-5 film (Eastman Kodak Co., Rochester, N.Y.).

**PCR.** After incubation and sufficient growth, the 21 strains were processed with proteinase K combined with buffer A (1 M Tris-HCl [pH 8.0], 1 M KCl, 1 M $MgCl_2$, Milli-Q distilled $H_2O$) and buffer B (1 M Tris-HCl [pH 8.0], 1 M $MgCl_2$, Triton X-100, Tween 20, Milli-Q distilled $H_2O$). One milliliter of culture was centrifuged for 20 min at 4°C, the supernatant was discarded, and the pellet was resuspended in proteinase K lysis buffer. The samples were incubated at

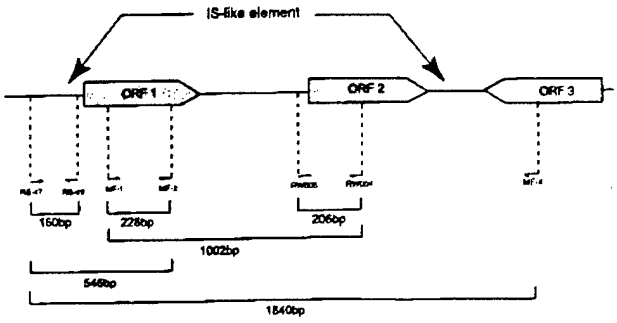


FIG. 2. Schematic diagram of the *M. fermentans* IS-like element and its flanking regions. The locations of all of the primer pairs used in this study are indicated, as are the sizes, in base pairs, of the respective amplicons. ORF-2 is the putative transposase. ORF-1 and ORF-3 have no assigned putative functions.

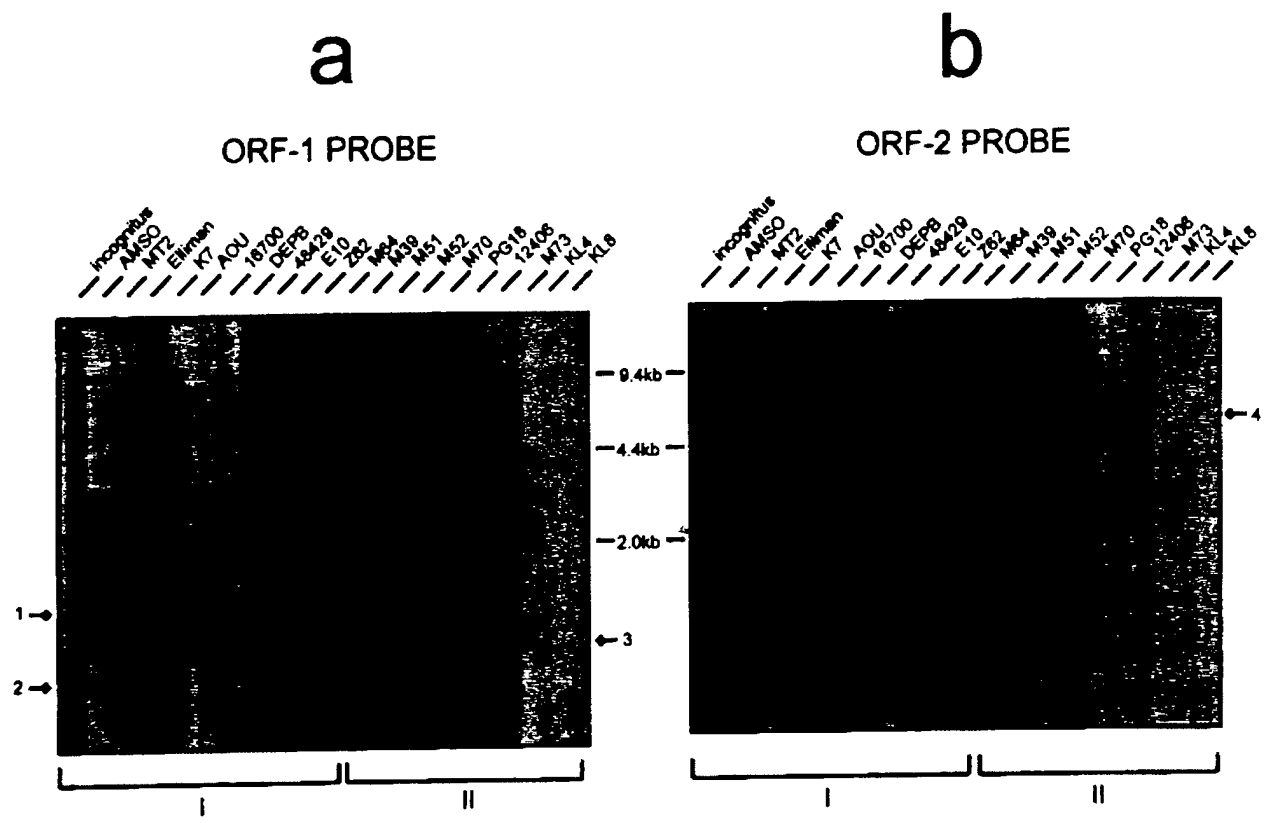


FIG. 3. Southern blot analysis of the chromosomal distribution of the *M. fermentans* IS-like element. DNAs isolated from all of the strains were digested with *Hin*dIII, separated in 0.8% agarose, transferred to nylon membranes, and then probed. The Southern blots were hybridized with an oligonucleotide specific for ORF-1 (a), and the same strains were then hybridized with an oligonucleotide specific for the transposase gene (b). Approximate fragment sizes are indicated in kilobases. Differences between the two basic genotypes (I and II) and the identification of fragments common to the two panels are indicated by the numbered arrows (see text).

60°C for 1 h and then boiled for 10 min. Samples were incubated on ice for 10 min and then stored at −70°C until ready for PCR.

Amplification of the *M. fermentans* strains was performed by using four different primer pairs (see Fig. 2). Primers RS-47 and RS-49 and primers RW005 and RW004 were previously described by S.-C. Lo et al. (34); primers MF-1 (5′-GGA AAA CTC TTA TTC AGC C-3′) and MF-2 (5′-GGA AAA CTC TTA TTC AGC ATG C-3′) were synthesized by Keystone Laboratories. Amplification of DNA was performed in a total volume of 50 μl. Basically, PCR was performed with 40 cycles of denaturation (94°C, 25 to 30 s), annealing (60°C, 1 min), and extension (72°C, 1 min). Another primer, MF-4 (5′-GCG GCA CCA TCA ATC ACA TAT AC-3′), was used as the antisense primer along with the previously described RS-47 sense primer. For this primer pair, an initial denaturation at 94°C for 2 min was followed by 40 cycles as described above. PCR products were resolved on 2% agarose gels and visualized by ethidium bromide staining.

**Immunoblotting.** SDS-polyacrylamide gel electrophoresis and Western blot analysis were performed as described previously, by using a 10% resolving gel and a 4% stacking gel, and then proteins were separated and transferred to nitrocellulose (Bio-Rad) by the method of Towbin et al. (33). Immunological reactions were visualized with peroxidase-labeled conjugates (Sigma).

**MAbs.** MAbs directed to *M. fermentans* incognitus antigens were produced in conjunction with the Hybridoma Core Facility of the Multipurpose Arthritis Center at the University of Alabama at Birmingham. The basic procedure for the production and characterization of MAbs has been described previously in detail (35).

## RESULTS AND DISCUSSION

**Phenotyping of *M. fermentans* strains and isolates.** The most frequently colonized sites in a mycoplasma-infected host are epithelial cell surfaces (7, 12, 14, 23, 31). In the case of *M. fermentans*, the second most frequent association is with blood cells (17, 21). Are there characteristics that make some species or some strains within a species uniquely qualified for survival in one site as opposed to another? We previously identified an *M. fermentans* surface antigen (Pra) that can be divided into two distinct domains based on the immunoblot pattern obtained with MAbs, i.e., a domain that is resistant to degradation by trypsin, chymotrypsin, V-8 protease, and proteinase K and a second domain that is sensitive to these same proteinases (40). Our preliminary studies suggest that Pra is a complex surface network consisting of acylated proteins, but the nature of the membrane anchor and the noncovalent forces that mediate the interaction between the two domains have not been fully characterized (39). Nonetheless, we hypothesized that a correlation exists between the expression of the proteinase-resistant domain, which may play a protective role at the organism surface, and the association of the organism with particular cell types. Results in Fig. 1 show the variable expression of the $Pra^+$ phenotype and the distinctive, diffuse immunoblot pattern of the $Pra^+$ phenotype which correlated with isolation of the organism associated with cells of bone marrow origin and frequently from immunocompromised patients (Table 1). This broad distribution of the electrophoretic mobility of identical epitopes seen for the $Pra^+$ phenotype is not uncommon for mycoplasmal antigens (38). The single exception to the above correlation was isolate 16700, which was isolated from the urethra of a patient with nongonococcal urethritis. Organisms lacking expression of the proteinase-resistant phenotype were most commonly isolated from the respiratory tract, from the genital tract, and from arthritic joints (Fig. 1 and Table 1). These Pra mutants were presumably epithelial cell associated. If the $Pra^+$ phenotype does, in fact, provide the organism with protection from proteolytic degradation, then the above correlations support the possibility, although they certainly do not prove, that the $Pra^+$ phenotype resides in a hydrolase-rich

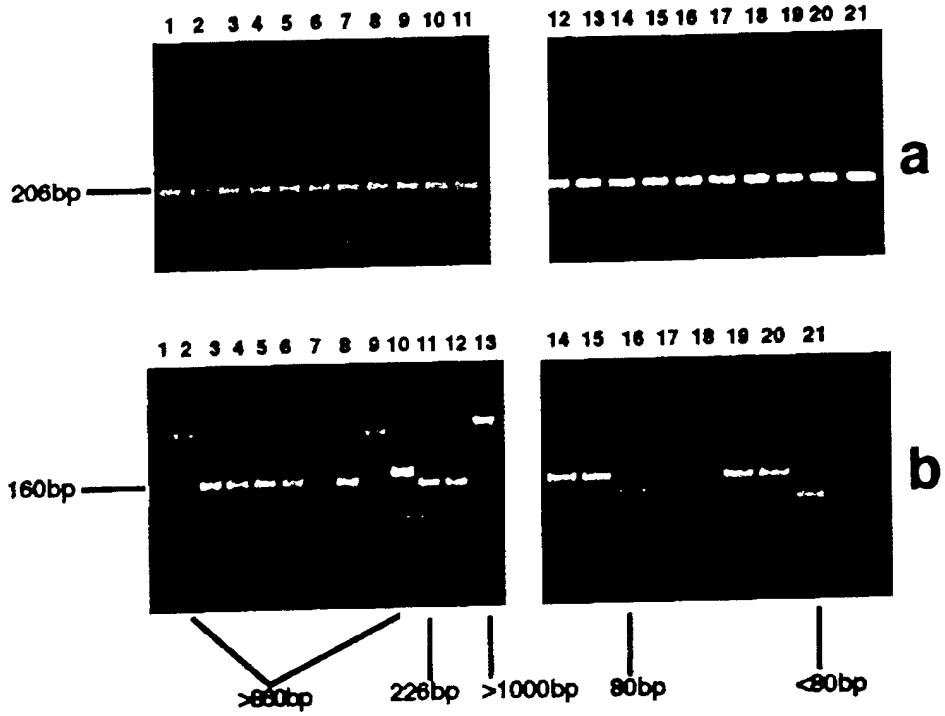


FIG. 4. PCR analysis of representative *M. fermentans* strains from various sources (Table 1), using primers RW005 and RW004 (a) and primers RS47 and RS49 (b), which are located within the transposase gene and upstream of the IS-like element, respectively (Fig. 2). PCR amplicons were analyzed by electrophoresis in a 2% agarose gel stained with ethidium bromide. Sizes of products are indicated. Lanes: 1, M51; 2, KL8; 3, AMSO; 4, Elliman; 5, MT2; 6, DEPB; 7, M52; 8, AOU; 9, KL4; 10, M64; 11, E10; 12, 16700; 13, 12406; 14, Z62; 15, incognitus; 16, M39; 17, M73; 18, PG18; 19, K7; 20, 48429; 21, M70.

intracellular compartment. This niche may be represented by the professional-phagocyte-rich cellular environment found in the circulation.

**Genomic distribution of the IS element.** Development of a statistically sound proof of the association suggested above requires a large number of isolates characterized with respect to Pra expression and sites of isolation. Therefore, if a genetic marker also existed (preferably associated with Pra in a non-dissociable genotype-phenotype relationship) which correlated with the site of isolation, then it might be possible to develop a more sensitive and simple system for discerning the Pra phenotype of new isolates. We initially looked for a correlation between Pra expression and the genomic location of the previously published *M. fermentans* IS-like element. Disruption or activation of cryptic promoters is not an uncommon result of IS movement (1, 4). Although it is unproved, *M. fermentans* may use this system to accommodate its surface properties for survival in its current environment (13). Figure 2 is a schematic showing the basic structure of the previously described IS-like element, as well as the locations of the genotyping PCR primers and probes used in the current study. Figure 3 shows the distribution of IS-associated ORF-1 and ORF-2 (transposase gene) in *Hin*dIII genomic digests of the different *M. fermentans* strains. These restriction patterns are consistent with those previously shown for strain incognitus (13). Based on the distribution of these two ORFs, the *M. fermentans* strains can be grouped into two basic genotypes (I and II). The published sequence of the incognitus strain IS-like element contains no *Hin*dIII site (13). If the IS-like elements in the other *M. fermentans* strains are sufficiently similar to the IS-like element of strain incognitus and are intact, then ORF-1- and ORF-2-specific probes should cohybridize to the same *Hin*dIII restriction fragments. Examination of Fig. 3 by using this criterion (i.e., identification of fragments common to Fig. 3a and b) indicates that genotype I has at least 10 copies of the intact IS and genotype II has only 3. These are minimal estimates, since a single fragment could contain multiple probe sites. Identification of fragments that are not common to Fig. 3a and b indicate that (i) all genotype I strains have one copy of ORF-1 which is not associated with an IS (Fig. 3a, location 1); (ii) two of the genotype I strains, incognitus and 16700, have one additional non-IS-associated ORF-1 (Fig. 3a, location 2); (iii) 7 of the 10 genotype II strains have one non-IS-associated ORF-1 (Fig. 3a, location 3); and (iv) 6 of the latter 7 genotype II strains also have a non-IS-associated ORF-2 (Fig. 3b, location 4). The apparent non-IS-associated ORFs in these experiments may be the result of a common mutational event producing a *Hin*dIII site between ORF-1 and ORF-2. Strains KL4 and KL8 are unusual, since no IS elements are detectable in Fig. 3. Even though the latter two strains may actually constitute a third genotype, here we have placed them into genotype II based on additional parameters to be discussed below.

Table 1 shows genotypes I and II in the context of Pra expression and site of isolation. All strains in genotype I were $Pra^+$, and all genotype II strains were Pra mutants.

**Genotyping of *M. fermentans* strains by PCR.** The pattern of distribution of the IS-like element genomic insertion sites was a reliable reflection of Pra expression but required very cumbersome methodology. The asymmetric hybridization of the ORF-1 and ORF-2 probes seen in Fig. 3 indicated the presence of some differences within the IS-like element which may provide useful sequence markers for determining genotypes by using PCR. An *M. fermentans*-specific PCR primer pair has been previously described (34). Amplification with this primer pair, located within ORF-2 of the IS-like element (Fig. 2, RW005 and RW004), resulted in a 206-bp amplicon for the

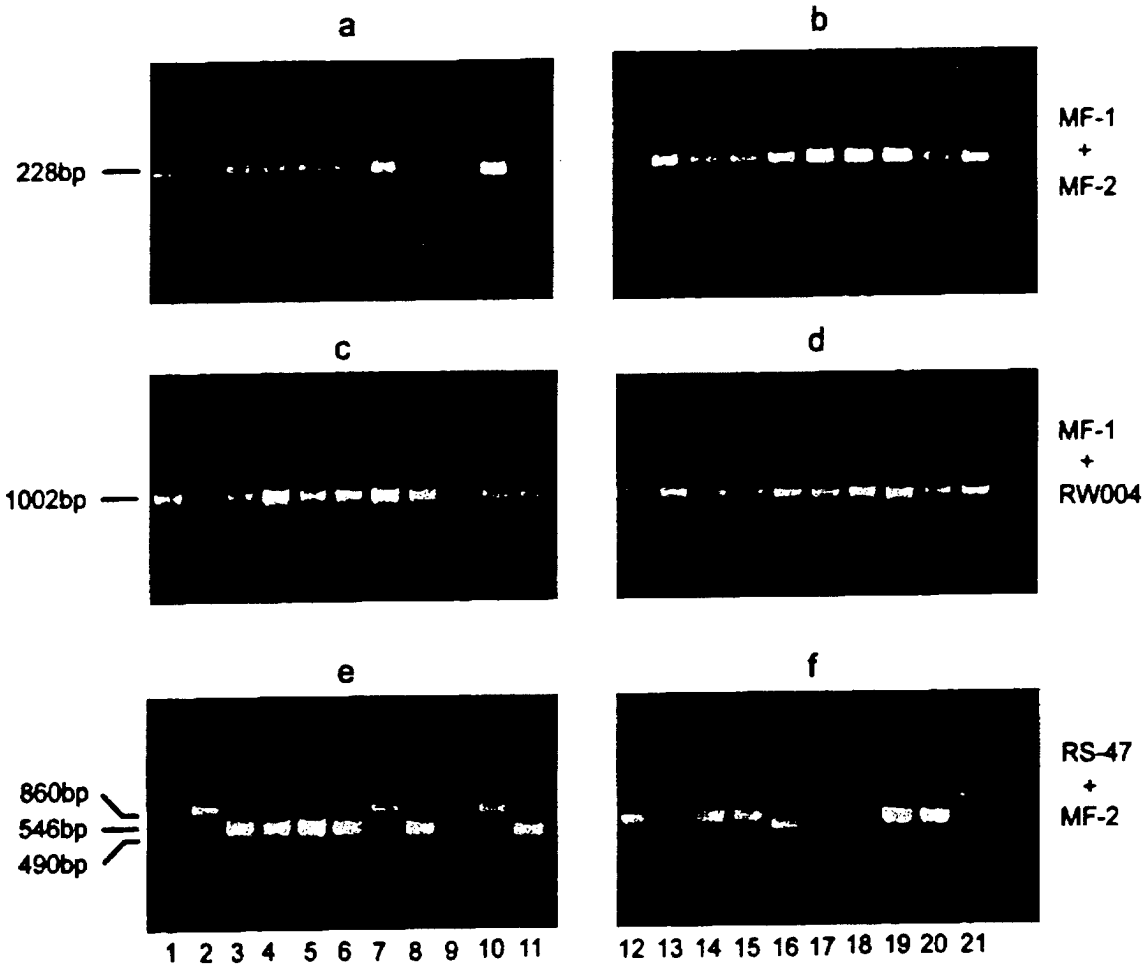


FIG. 5. PCR analysis of *M. fermentans* strains. Amplification of representative *M. fermentans* strains from various sources (Table 1) by using primers MF-1 and MF-2, MF-1 and RW004, and RS-47 and MF-2 (Fig. 2) is shown. PCR amplicons were analyzed by electrophoresis in 2% agarose gels and stained with ethidium bromide. Lanes in a, c, and e: 1, M51; 2, KL8; 3, AMSO; 4, Elliman; 5, MT2; 6, DEPB; 7, M52; 8, AOU; 9, KL4; 10, M64; 11, E10. Lanes in b, d, and f: 12, 16700; 13, 12406; 14, Z62; 15, incognitus; 16, M39; 17, M73; 18, PG18; 19, K7; 20, 48429; 21, M70. Sizes of products are indicated on the left.

strains evaluated in that study. In the current study, amplification with these same primers produced a 206-bp amplicon for all of the strains listed in Table 1 (Fig. 4a). Although these primers performed as predicted, they do not allow discrimination of the two genotypes. Strains KL4 and KL8 showed no indication for the presence of either ORF-1 or ORF-2 (Fig. 3), even though a typical 206-bp product was obtained with the RW005-RW004 primer pair (Fig. 4a). This suggested that even though the RW005 and RW004 primer sites were present, the intervening sequence was sufficiently different to disallow hybridization of the RW006 probe. This may result in a dysfunctional transposase, which could explain the absence of multiple insertion sites in these two strains.

In the same previous study as that described above, another primer pair located immediately upstream of the IS-like element (Fig. 2, RS47 and RS49) produced a 160-bp product for only three of the six strains tested. The RS47-RS49 primer pair was not further evaluated, since those investigators were interested in defining PCR primers for detection of all strains of *M. fermentans*. When we evaluated the strains listed in Table 1 with the RS47-RS49 primer pair, the most common product was 160 bp (Fig. 4b). Other products ranged from ≤80 bp (M39 and M70) to over ≥860 bp (KL8, KL4, and 12406). Some strains also gave no distinct product (M51, M52, M73, and PG18). These results are recorded in Table 1, and there is a complete correlation between genotype I, Pra expression, and the IS-like element location resulting in a 160-bp product with the RS primers. PCR amplification of all genotype II strains resulted in different-sized amplicons or no amplicon. The lack of an RS product for strains M51, M52, M73, and PG18 implies that either one or both primer sites are missing (or lack sufficient homology) or that there is an insertion between the primer sites resulting in a template which was too large to amplify efficiently.

Various PCR primer combinations (Fig. 2) were used to determine if there were any differences in the sequence within the IS-like element which might allow the detection of a stable PCR product that would be representative of the genotype II strains. Amplification with the primers MF-1 and MF-2 suggested that ORF-1 was not significantly different among the strains (Fig. 5a and b). Strains KL4 and KL8 gave no product, and strain 16700 consistently produced a weak amplicon. Similarly, by using primers MF-1 and RW004, we found no differences in the linkage between ORF-1 and ORF-2 among the strains (Fig. 5c and d). Once again, strains KL4 and KL8 gave no product and strain 16700 was amplified poorly. The linkage between the RS genomic site and the ORF-1 IS site was evaluated by using primers RS-47 and MF-2 (Fig. 5e and f). Am-

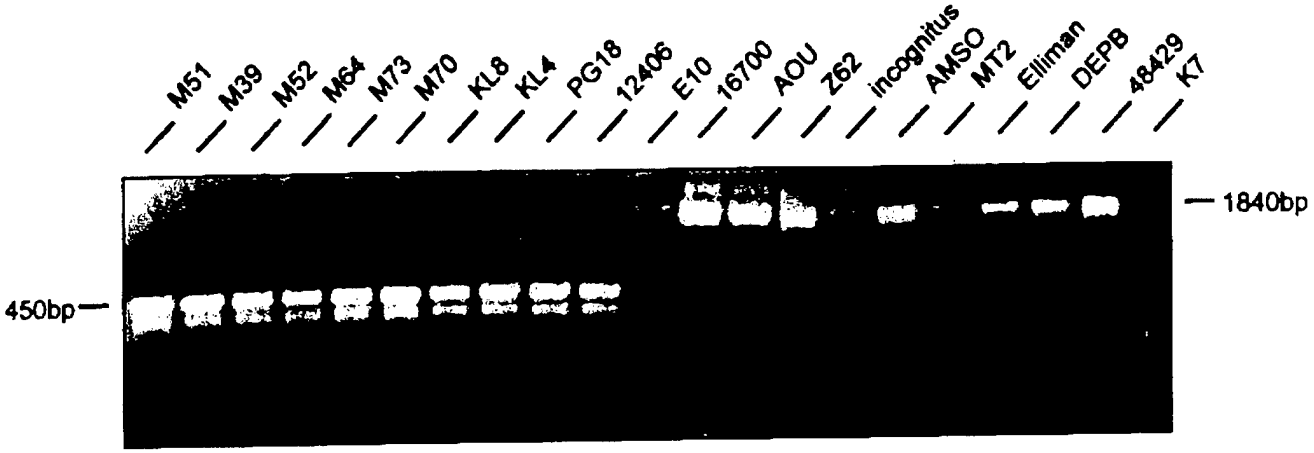


FIG. 6. PCR analysis of the *M. fermentans* strains by using primer sites which flank the IS-like element. Primer pair RS-47–MF-4 (Fig. 2) amplicons were analyzed by electrophoresis in a 2% agarose gel and stained with ethidium bromide. Sizes of products are indicated. Genotype II strains are represented by the 450-bp product. Genotype I strains were not consistently amplified.

plification of all genotype I strains resulted in a 546-bp amplicon, reaffirming the stability of this particular insertion site for the genotype I strains. The genotype II strains were inconsistently amplified with this primer pair, indicating the instability of this insertion site in these strains.

Since Fig. 4b, 5e, and 5f suggested that the genotype II strains do not have an IS-like element consistently present near the RS site, we used primers RS-47 and MF-4, which flank the putative insertion site, to ascertain if the IS was consistently absent from this site. Figure 6 shows a stable 450-bp product for all genotype II strains, indicating that the genotype II strains have a stable linkage between the RS site and ORF-3 with no intervening IS-like element.

These analyses indicate that the two *M. fermentans* genotypes consistently differed in the sites of insertion of the IS-like element but not in the sequence of the element itself. Also, due to potential sequence instability within the IS-like element, the only r· able genotyping markers reside outside the IS-like eleme and in the genomic sequences immediately upstream and downstream of the IS junctions. Therefore, the method of choice for species detection appears to be direct detection of specific sequences within the IS-like element. In contrast, for genotype distinction, detection of stable genomic insertion sites is required, i.e., with primers RS47 and RS49 for genotype I and primers RS47 and MF-4 for genotype II. These primer pairs should allow easy and rapid genotyping of *M. fermentans* in clinical samples and thus obviate the need for the frequently unsuccessful isolation and culturing of this organism.

Aside from the immediate usefulness for diagnostic genotyping, these data also will help to understand how this species may adapt for survival in a particular host population. At this time, we have only a suggestive link between the sites of insertion of the IS-like element and Pra expression, and as previously stated by Lo et al. (13), there is no proof that this element is mobile. Conclusive evidence will have to await a more direct connection between the *pra* gene sequence and a specific insertion site. We have not analyzed a sufficient number of isolates to say that proteinase resistance is always associated with isolation from cells of a blood-related compartment or from immunocompromised patients, but completion of these analyses will determine if *M. fermentans* uses the mobility of this IS-like element coupled to Pra expression as a primary means of maintaining a specific niche in its host.

## ACKNOWLEDGMENTS

This research was supported by grant DAMD17-97-1-7001 from the Department of the Army and grant 5R01 AI33197 from the National Institutes of Health. MAbs were produced in conjunction with the Hybridoma Core Facility of the Multipurpose Arthritis Center at the University of Alabama at Birmingham, which is supported by grant 2P60 AR0614-20 from the National Institutes of Health.

## REFERENCES

1. **Barnett, M. J., B. G. Rushing, R. F. Fisher, and S. R. Long.** 1996. Transcription start sites for *syrM* and *nodD3* flank an insertion sequence relic in *Rhizobium meliloti*. J. Bacteriol. **178**:1782–1787.
2. **Bartholomew, L. E.** 1967. Characterization of mycoplasma strains and antibody studies from patients with rheumatoid arthritis. Ann. N. Y. Acad. Sci. **143**:522–534.
3. **Biberfeld, G., and E. Nilsson.** 1978. Mitogenicity of *Mycoplasma fermentans* for human lymphocytes. Infect. Immun. **21**:48–54.
4. **Brikun, I., K. Suziedelis, O. Stemmann, R. Zhong, L. Alikhanian, E. Linkova, A. Mironov, and D. E. Berg.** 1996. Analysis of CRP-CytR interactions at the *Escherichia coli udp* promoter. J. Bacteriol. **178**:1614–1622.
5. **Capobianchi, M. R., G. Lorino, M. T. Lun, C. Mancini, P. Di Marco, and F. Dianzani.** 1987. Membrane interactions involved in the induction of interferon-alpha by Mycoplasma pneumoniae. Antiviral Res. **8**:115–124.
6. **Cassell, G. H., J. W. Clyde, Jr., and J. K. Davis.** 1985. Mycoplasmal respiratory infections, p. 65–106. *In* S. Razin and M. F. Barile (ed.), The mycoplasmas, vol. 4. Mycoplasma pathogenicity. Academic Press, Inc., New York, N.Y.
7. **Chen, Y. Y., and D. C. Krause.** 1988. Parasitism of hamster trachea epithelial cells by *Mycoplasma pneumoniae*. Infect. Immun. **56**:570–576.
8. **Chowdhury, I. H., T. Munakata, Y. Koyanagi, S. Kobayashi, S. Arai, and N. Yamamoto.** 1990. Mycoplasma can enhance HIV replication in vitro: a possible cofactor responsible for the progression of AIDS. Biochem. Biophys. Res. Commun. **170**:1365–1370.
9. **Gabridge, M. G., G. D. Abrams, and W. H. Murphy.** 1972. Lethal toxicity of Mycoplasma fermentans for mice. J. Infect. Dis. **125**:153–160.
10. **Gabridge, M. G., and D. D. Gamble.** 1974. Independence of leukemoid potential and toxigenicity of Mycoplasma fermentans. J. Infect. Dis. **130**: 664–668.
11. **Halden, N. F., J. B. Wolf, and W. J. Leonard.** 1989. Identification of a novel site specific endonuclease produced by Mycoplasma fermentans: discovery while characterizing DNA binding proteins in T lymphocyte cell lines. Nucleic Acids Res. **17**:3491–3499.
12. **Hu, P. C., U. Schaper, A. M. Collier, W. A. Clyde, Jr., M. Horikawa, Y. S. Huang, and M. F. Barile.** 1987. A *Mycoplasma genitalium* protein resembling the *Mycoplasma pneumoniae* attachment protein. Infect. Immun. **55**:1126–1131.
13. **Hu, W. S., R. Y. Wang, R. S. Liou, J. W. Shih, and S. C. Lo.** 1990. Identification of an insertion-sequence-like genetic element in the newly recognized human pathogen Mycoplasma incognitus. Gene **93**:67–72.
14. **Izumikawa, K., D. K. Chandler, M. W. Grabowski, and M. F. Barile.** 1987. Attachment of Mycoplasma hominis to human cell cultures. Isr. J. Med. Sci. **23**:603–607. (Erratum, **23**:868, 1987.)

15. **Kirchner, H., A. Bauer, T. Moritz, and F. Herbst.** 1986. Lymphocyte activation and induction of interferon gamma in human leukocyte cultures by the mitogen in Mycoplasma arthritidis supernatant (MAS). Scand. J. Immunol. **24**:609–613.
16. **Lemaitre, M., Y. Henin, F. Destouesse, C. Ferrieux, L. Montagnier, and A. Blanchard.** 1992. Role of mycoplasma infection in the cytopathic effect induced by human immunodeficiency virus type 1 in infected cell lines. Infect. Immun. **60**:742–748.
17. **Lo, S., M. Dawson, P. B. Newton III, M. Sonoda, J. Shih, W. Engler, R. Wang, and D. Wear.** 1989. Association of the virus-like infectious agent originally reported in patients with AIDS with acute fatal disease in previously healthy non-AIDS patients. Am. J. Trop. Med. Hyg. **41**:364–376.
18. **Lo, S.-C.** 1986. Isolation and identification of a novel virus from patients with AIDS. Am. J. Trop. Med. Hyg. **35**:675–676.
19. **Lo, S.-C., R. Wang, P. Newton, N. Yang, M. Sonoda, and J. Shih.** 1989. Fatal infection of silvered leaf monkeys with a virus-like infectious agent (VLIA) derived from a patient with AIDS. Am. J. Trop. Med. Hyg. **40**:399–409.
20. **Lo, S. C., M. S. Dawson, D. M. Wong, P. B. Newton III, M. A. Sonoda, W. F. Engler, R. Y. Wang, J. W. Shih, H. J. Alter, and D. J. Wear.** 1989. Identification of Mycoplasma incognitus infection in patients with AIDS: an immunohistochemical, in situ hybridization and ultrastructural study. Am. J. Trop. Med. Hyg. **41**:601–616.
21. **Lo, S. C., J. W. Shih, P. B. Newton III, D. M. Wong, M. M. Hayes, J. R. Benish, D. J. Wear, and R. Y. Wang.** 1989. Virus-like infectious agent (VLIA) is a novel pathogenic mycoplasma: Mycoplasma incognitus. Am. J. Trop. Med. Hyg. **41**:586–600.
22. **Lo, S. C., S. Tsai, J. R. Benish, J. W. Shih, D. J. Wear, and D. M. Wong.** 1991. Enhancement of HIV-1 cytocidal effects in $CD4^+$ lymphocytes by the AIDS-associated mycoplasma. Science **251**:1074–1076.
23. **Morrison-Plummer, J., A. Lazzell, and J. B. Baseman.** 1987. Shared epitopes between *Mycoplasma pneumoniae* major adhesin protein P1 and a 140-kilodalton protein of *Mycoplasma genitalium*. Infect. Immun. **55**:49–56.
24. **Murphy, W. H., C. Bullis, L. Dabich, R. Heyn, and C. J. Zarafonetis.** 1970. Isolation of mycoplasma from leukemic and nonleukemic patients. J. Natl. Cancer Inst. **45**:243–251.
25. **Murphy, W. H., I. J. Ertel, and C. J. Zarafonetis.** 1965. Virus studies of human leukemia. Cancer **18**:1329–1344.
26. **Plata, E. J., M. R. Abell, and W. H. Murphy.** 1973. Induction of leukemoid disease in mice by Mycoplasma fermentans. J. Infect. Dis. **128**:588–597.
27. **Quentmeier, H., E. Schmitt, H. Kirchhoff, W. Grote, and P. F. Mühlradt.** 1990. *Mycoplasma fermentans*-derived high-molecular-weight material induces interleukin-6 release in cultures of murine macrophages and human monocytes. Infect. Immun. **58**:1273–1280.
28. **Root-Bernstein, R. S., and S. H. Hobbs.** 1991. Homologies between mycoplasma adhesion peptide, CD4 and class II MHC proteins: a possible mechanism for HIV-mycoplasma synergism in AIDS. Res. Immunol. **142**: 519–523.
29. **Saillard, C., P. Carle, J. M. Bove, C. Bebear, S. C. Lo, J. W. Shih, R. Y. Wang, D. L. Rose, and J. G. Tully.** 1990. Genetic and serologic relatedness between Mycoplasma fermentans strains and a mycoplasma recently identified in tissues of AIDS and non-AIDS patients. Res. Virol. **141**:385–395.
30. **Stuart, P. M., G. H. Cassell, and J. G. Woodward.** 1989. Induction of class II MHC antigen expression in macrophages by Mycoplasma species. J. Immunol. **142**:3392–3399.
31. **Taylor-Robinson, D.** 1989. Genital mycoplasma infections. Clin. Lab. Med. **9**:501–523.
32. **Taylor-Robinson, D., R. H. Purcell, D. C. Wong, and R. M. Chanock.** 1966. A colour test for the measurement of antibody to certain mycoplasma species based upon the inhibition of acid production. J. Hyg. **64**:91–104.
33. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
34. **Wang, R. Y., W. S. Hu, M. S. Dawson, J. W. Shih, and S. C. Lo.** 1992. Selective detection of *Mycoplasma fermentans* by polymerase chain reaction and by using a nucleotide sequence within the insertion sequence-like element. J. Clin. Microbiol. **30**:245–248.
35. **Watson, H. L., L. S. McDaniel, D. K. Blalock, M. T. Fallon, and G. H. Cassell.** 1988. Heterogeneity among strains and a high rate of variation within strains of a major surface antigen of *Mycoplasma pulmonis*. Infect. Immun. **56**:1358–1363.
36. **Williams, M. H., J. Brostoff, and I. M. Roitt.** 1970. Possible role of Mycoplasma fermentans in pathogenesis of rheumatoid arthritis. Lancet **ii**:277–280.
37. **Williamson, J. S., J. K. Davis, and G. H. Cassell.** 1986. Polyclonal activation of rat splenic lymphocytes after in vivo administration of *Mycoplasma pulmonis* and its relation to in vitro response. Infect. Immun. **52**:594–599.
38. **Wise, K. S., D. Yogev, and R. Rosengarten.** 1992. Antigenic variation, p. 473–489. *In* J. Maniloff, R. N. McElhaney, L. R. Finch, and J. B. Baseman (ed.), Mycoplasmas: molecular biology and pathogenesis. American Society for Microbiology, Washington, D.C.
39. **Zuhua, C., G. H. Cassell, and H. L. Watson.** 1992. A major membrane antigen complex of *Mycoplasma fermentans* contains multiple, linked domains, abstr. PS1/94, p. 227. *In* Program and abstracts of the 9th Congress of the International Organization for Mycoplasmology 1992. International Organization for Mycoplasmology, Ames, Iowa.
40. **Zuhua, C., G. H. Cassell, and H. L. Watson.** 1992. Protease resistance of Mycoplasma fermentans surface antigens, abstr. PS1/95, p. 228. *In* Program and abstracts of the 9th Congress of the International Organization for Mycoplasmology 1992. International Organization for Mycoplasmology, Ames, Iowa.

disease activity in rheumatoid arthritis and os-
39–148.
S. MATT, D. YOCUM & L. CASTRO. 1997. Exam-
s as a factor in disease exacerbations among
hav. Med. **19:** 279–286.
J. COHEN & M. STEIN. 1986. Major depressive
atry **46:** 81–87.
v-Hill Book Company. New York.
OGROCKI, J. C. STOUT, C. E. SPEICHER & R.
isruption, and immune function. Psychosom.

concepts of stress and stress system disorders.
eostasis. J. Am. Med. Assoc. **267:** 1244–1252.
SON & P. J. O'HARE. 1993. How perceived con-
heumatoid arthritis patients. J. Soc. Clin. Psy-

ZAUTRA & S. ROTH. 1992. Effects of stress on
osteoarthritis. Arthritis Rheum. **35(4 Suppl.):**

MEZ-SANCHEZ, L. H. SILVEIRA, P. MARTINEZ-
. 1991. Prolactin, immunoregulation, and au-
: 273–284.

---

*F THE PAPER*

*ondon, United Kingdom*): I would worry
nout the objective evidence of disease ac-
y to the highly subjective outcome mea-
t. Objective evidence of disease activity
t count, and so forth.

*ty, Tempe, Arizona*): That is an excellent
t altogether objective.

*College of Medicine, Peoria, Illinois*): Do
racterization of sleep as it relates to the

than measure fatigue, and I do not have
ly is an interesting point.
relationships between sleep quality and
sitivity.

# Stress and Immune Dysfunction in Gulf War Veterans[a]

MICHAEL P. EVERSON,[b] SUSAN KOTLER, AND WARREN D. BLACKBURN, JR.

*Veterans Affairs Medical Center and The University of Alabama at Birmingham, Birmingham, Alabama 35233, USA*

**ABSTRACT: The role of stress and immunological abnormalities, as well as the neuroendocrine regulation of these two variables, in illnesses in Gulf War veterans (GWVs) is unknown. Many GWV patients complain of skin and joint problems, that is, disorders that may have a common immunological basis. Post-traumatic stress disorder (PTSD), an anxiety disorder associated with chronic stress, is diagnosed in approximately 10% of the Alabama GWVs. Chronic stress can lead to a reduced capacity to resist disease. Recent work suggests that a dysregulated balance of cytokines produced by T helper cells of the immune system can play a significant role in stress-related illnesses. Indeed, a balanced immune response (cell-mediated and humoral immunity) is an important defense mechanism, and cytokines can regulate this balance. It is therefore plausible that stress-induced changes in hormones (such as cortisol and catecholamines) and cytokines, both of which have been implicated in altering levels of cellular or humoral immunity, may play a role in GWV illnesses.**

Approximately 697,000 United States military personnel were deployed to the Persian Gulf to participate in Operation Desert Shield/Desert Storm during the latter months of 1990 and early months of 1991. After returning from the Gulf War, many Gulf War veterans began reporting a variety of symptoms which they suspected to be associated with military service during the Gulf War. These symptoms included fatigue, headaches, gastrointestinal distress, skin problems, musculoskeletal complaints, sleep disturbances, and cognitive dysfunction. These are the same types of symptoms that have been compiled in the Department of Defense (DoD) and Veterans Affairs (VA) Gulf War registries.[1] Kroenke *et al.* recently reported that in 18,495 Gulf War veterans, joint pain, fatigue, rash, and sleep disturbances were among the most common symptoms reported, that is, disorders which may have a common immunological basis and/or a rheumatic component. To explain the etiological basis for these complaints, many hypotheses have been put forth including exposure to infectious agents, chemical warfare agents, vaccines, and environmental pollutants. To date, none of these hypotheses has been proven.

An alternate hypothesis to explain these undiagnosed conditions in Gulf War veterans is that they are stress-related illnesses that are associated with the physiological consequences of stressful combat and/or post-traumatic stress disorder (PTSD). In-

[a]This work was supported by funds from the Department of Defense grant DoD-42.

[b]Address for correspondence: VA Medical Center (111F), 700 South 19th Street, Birmingham, AL 35233. Tel: (205) 934-4212; fax: (205) 934-4198; e-mail: meverson@uab.edu

Is There Immune Dysregulation in Symptomatic Gulf War Veterans? Michael P. Everson, Ke Shi, Peggy Aldridge, Alfred A. Bartolucci, and Warren D. Blackburn, Jr. VA Medical Center and UAB, Birmingham, Alabama, USA.

Many Gulf War veterans complain of a variety of symptoms including skin rashes and joint pain which may have a common immunological basis. Other Gulf War veterans have post-traumatic stress disorder (PTSD), an anxiety disorder associated with chronic stress. Chronic stress maybe associated with a reduced capacity to resist disease, and recent work suggests that a dysregulated balance of cytokines produced by T helper cells of the immune system can play a significant role in stress-related illnesses. It is known that a balanced immune response (cell-mediated and humoral immunity) is an important defense mechanism. Although the mechanism by which a change in immune system balance occurs is not clear, it may be secondary to stress-induced changes in hormones such as cortisol and catecholamines, both of which have been implicated in altering levels of cellular or humoral immunity. For these reasons, we are investigating the function of both the cellular and humoral arms of the immune system as well as the cytokine patterns associated with these different functions.

The goal of our study is to determine whether Gulf War veterans with a variety of symptoms, some of which are rheumatic in nature, have alterations in their immunological status. The study groups are symptomatic Gulf War veterans with unexplained symptoms involving two or more organ systems and two control groups consisting of asymptomatic Gulf War veterans and non-Gulf War veterans who have applied for disability compensation, i.e., have symptoms but did not participate in the Gulf War. Gulf War veterans with a PTSD diagnosis or other diagnosis which could explain their symptoms have been excluded from this analysis as a confounding variable.

*In vitro* immune function studies were used to compare the symptomatic Gulf War veterans and control groups. We have tested 3 doses of PHA and Con A as mitogenic stimulants and 3 doses of tetanus toxoid, anthrax vaccine, and *Candida albicans* as recall antigenic stimulants in proliferative cultures and recorded stimulation indices for each stimulant. We also tested natural killer activity using a non-radioactive procedure. Statistical comparisons were performed using analysis of variance and post-hoc analysis for pairwise comparisons. Natural killer cell activity was lower in symptomatic patients and disability controls relative to asymptomatic controls but these data did not reach statistical significance. No statistical differences ($p > 0.05$) were found between the groups when dose-response data for mitogens and antigens were analyzed or when magnitude of mitogenic or antigenic responses were analyzed. These data suggest that the proliferative response of T lymphocytes to non-specific mitogenic signals and specific antigenic signals is not impaired in symptomatic Gulf War veteran patients relative to asymptomatic and disability controls. We are currently preparing samples for batch cytokine analyses. It is expected that the cytokine data may yield further information relative to the neuroendocrine immune loop in these veterans.